

Reliability of the E-Test Method for Detection of Colistin Resistance in Clinical Isolates of *Acinetobacter baumannii*

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We compared the E-test to the broth microdilution method for testing the susceptibility of 115 clinical isolates of *Acinetobacter baumannii* to colistin. Twenty-two (19.1%) strains were resistant to colistin and 93 (80.8%) strains were susceptible according to the reference broth microdilution method. A categorical agreement of 98.2% was found, with only two (1.7%) very major errors. Agreement within 1 twofold dilution between the E-test and the broth microdilution was 16.5%. Complete agreement was found for the strains for which MICs fell within the range of 0.25 to 1 µg of colistin/ml. However, there was poor concordance, particularly in extreme dilutions with higher MICs by the E-test method.

Acinetobacter baumannii has emerged as an important nosocomial pathogen (3), particularly for patients admitted to an intensive-care unit. Treatment of these infections is often difficult, because clinical isolates of *A. baumannii* are generally resistant to multiple antibiotics (7, 10, 13).

The clinical use of polymyxins has been limited to topical formulations for the treatment of several diseases, and they have been used prophylactically for the prevention of infection in neutropenic or cystic fibrosis patients (5). Recently, the potential therapeutic indication for the parenteral use of polymyxins has been restored for the treatment of multidrug-resistant *Acinetobacter* sp. isolates causing life-threatening infections (9, 12).

The in vitro (1, 4) and in vivo activities of colistin (polymyxin E) suggested that it would be an effective antimicrobial agent against *A. baumannii* (6, 9, 11, 12, 18). However, some polymyxin-resistant strains have been reported (15, 16).

Problems in performing in vitro colistin susceptibility testing of this organism have also been encountered. The disk diffusion method has been found to be inaccurate and not reproducible for the testing of *A. baumannii* (8). Agar dilution and broth microdilution (BMD) are the currently recommended susceptibility test methods for this organism, but they are cumbersome to perform and impractical to implement as routine tests in many clinical laboratories (8, 14).

The E-test (AB Biodisk, Solna, Sweden) has been reported to be a simple and accurate alternative method for determining the antimicrobial susceptibilities of various microorganisms, including fastidious bacteria, although no experience with this method for testing the susceptibility of *A. baumannii* to colistin has yet been reported. We evaluated the accuracy of the E-test, compared to that of the BMD reference method, for testing the susceptibility of *A. baumannii* to colistin and its reliability for the detection of colistin resistance in clinical isolates of *A. baumannii*.

(Part of this work was presented at the 13th European Congress of Clinical Microbiology and Infectious Diseases, Glasgow, United Kingdom, 10 to 13 May 2003 [2].)

A total of 115 clinical isolates of *A. baumannii* for which a wide range of colistin MICs (0.03 to 512 µg/ml) had been found were studied. They were randomly chosen during the past 7 years and originated from several sources: blood cultures (31 isolates), bronchial aspirates (23 isolates), cerebrospinal fluids (15 isolates), carriage controls (12 isolates), wound infections (12 isolates), skin ulcers (9 isolates), catheters (7 isolates), surgical drains (2 isolates), conjunctival exudate (1 isolate), nasal exudate (1 isolate), peritoneal fluid (1 isolate), and an intraabdominal abscess (1 isolate). *A. baumannii* was identified by Gram stain appearance, colonial morphology, motility, cytochrome oxidase reaction, and growth at 44°C, as well as by the semiautomated MicroScan Walk Away method (Dade-Behring). All the strains were confirmed as *A. baumannii* by amplified ribosomal rRNA gene restriction analysis (ARDRA) (17). The isolates were stored at –70°C until the susceptibility testing assays were performed. *Escherichia coli* ATCC 25922 (β-lactamase negative) was included as a quality control (QC) strain in both susceptibility testing methods.

Colistin sulfate powder was obtained from Sigma Chemical Co. (St. Louis, Mo). Susceptibility testing by BMD with cation-adjusted Mueller-Hinton broth (BBL-Becton Dickinson) was carried out in accordance with NCCLS guidelines and was used as the reference method (14). Briefly, inocula were prepared by suspending colonies from overnight growth plates in 5 ml of sterile saline to match the turbidity of a 0.5 McFarland standard for a final inoculum of 5×10^5 CFU/ml. Colistin concentrations from 0.015 to 1,024 µg/ml were tested in microtiter trays. The MIC was defined as the lowest concentration with which no growth was visible after incubation of plates at 35°C for 16 to 20 h.

MICs were determined by the E-test method according to the manufacturer's guidelines (AB Biodisk). A suspension of each isolate in Mueller-Hinton broth, adjusted to the density of a 0.5 McFarland standard, was swabbed in three directions to ensure uniform growth onto Mueller-Hinton agar plates. Once

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TABLE 1. Distribution of differences in MICs of colistin against 115 *A. baumannii* isolates by the E-test versus the BMD reference method

No. of isolates with a MIC difference ^a of:							% Agreement within 1 log ₂ dilution
<-2	-2	-1	0	+1	+2	>+2	
5	4	6	6	7	24	63	16.5

^a Expressed in log₂ dilutions.

the agar surface was completely dry, an E-test colistin strip (ranging from 0.06 to 1,024 µg/ml) was applied to each plate with sterile forceps, and the plates were incubated at 35°C for 16 to 20 h. The MIC was read where inhibition of growth intersected the E-test strip. When small colonies grew within the zone of inhibition or a haze of growth occurred around MIC end points, the highest MIC intersect was recorded. For comparison of the results of the two methods, E-test MICs were rounded up to the next higher twofold dilution. Because 0.06 µg/ml was the lowest colistin concentration tested by the E-test, reference MICs of ≤0.03 µg/ml were recorded as ≤0.06 µg/ml to ensure equal precision in comparison of the two susceptibility testing methods. The two susceptibility tests were performed simultaneously.

Because NCCLS documents do not provide interpretative criteria, a ≥4-µg/ml colistin concentration was used as the breakpoint to designate resistant isolates (8). Agreement between the two methods was defined as MICs that differed by ±1 log₂ dilution or less. Categorical agreement was defined as test results within the same susceptibility categories. Errors were ranked as follows: very major error, the reference method result was resistant and the E-test result was susceptible (false-susceptible result); major error, the reference method result was susceptible and the E-test result was resistant (false-resistant result). The usefulness of the E-test method (sensitivity, specificity, positive and negative predictive values) for the detection of colistin resistance in clinical isolates of *A. baumannii* was also evaluated.

All 115 strains classified by phenotypic and growth tests as *A. baumannii* or *Acinetobacter haemolyticus* were identified as *A. baumannii* (genomospecies 2) by the ARDRA genotypic method. Twenty-two (19.1%) out of 115 *A. baumannii* strains were resistant to colistin, and the remaining 93 (80.9%) strains were susceptible, by the BMD reference method. MICs for the *E. coli* QC strain were within the established reference MIC range (0.125 to 0.5 µg/ml), with values of 0.25 (by the BMD reference method) and 0.5 µg/ml (by E-test).

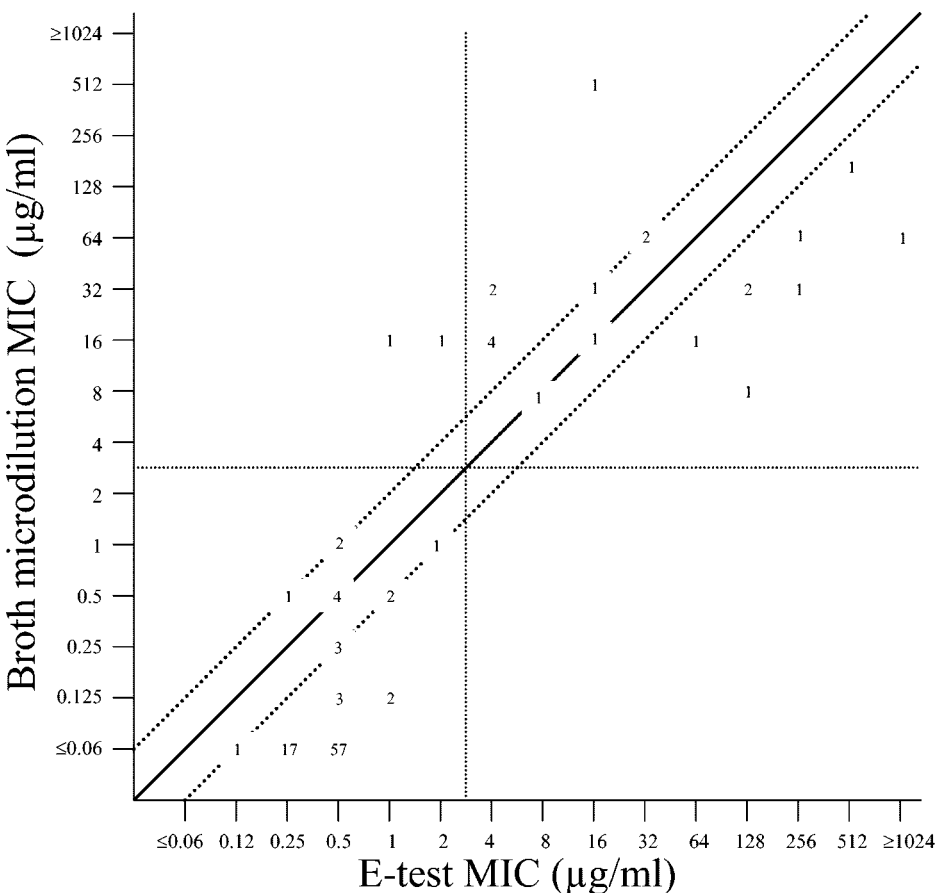


FIG. 1. Scattergram results comparing colistin MICs determined by the E-test with those determined by the BMD reference method ($n = 115$). The diagonal black line represents complete agreement, and the numbers represent the occurrences observed at each point. The broken lines represent ± 1 -log₂ MIC agreement limits between test results. Horizontal and vertical broken lines indicate the resistant MIC breakpoint (≥ 4 µg/ml).

The colistin MICs at which 50 and 90% of isolates were inhibited were 0.06 and 32 µg/ml, respectively, by BMD and 0.5 and 16 µg/ml, respectively, by E-test. Agreement within 1 twofold dilution between the E-test and the BMD reference method was 16.5% (19 of 115 strains). The worst agreement was found mainly with strains for which MICs ranged from ≤0.06 to 0.25 µg/ml and from 64 to ≥1,024 µg/ml by the reference method. This fact could be related to the poor diffusion of polymyxins in agar (8). The distribution of differences in MICs determined by the two methods is presented in Table 1. A scattergram comparing E-test MICs with MICs determined by the BMD reference method (Fig. 1) showed a trend toward higher MICs by the E-test method in extreme dilutions, particularly at low concentrations. Complete agreement was found with the strains for which colistin MICs were within the range of 0.25 to 1 µg/ml.

The E-test was read according to the manufacturer's instructions, which specified reading at the point of complete inhibition of all growth, including hazes. However, the MICs obtained by the reference method were significantly nearer to the reading point excluding hazes. This phenomenon was present only for 9 out of 22 resistant strains, and the hazes were cultured and incubated for 4 days with no growth resulting.

All 93 (100%) strains defined as susceptible by BMD were determined to be susceptible by the E-test. The E-test classified as colistin resistant 20 (90.9%) of the 22 strains defined as resistant by BMD. Overall, two very major errors were found (1.7%), but no major error was detected. The sensitivity of the E-test was 90.9%, and its specificity was 100%; the positive and negative predictive values were 100 and 97.8%, respectively.

The objective of the study was to assess the accuracy of the E-test, compared with that of the BMD method, for testing the susceptibility of *A. baumannii* to colistin and its reliability for the detection of colistin resistance among clinical isolates of *A. baumannii*. The E-test was a useful method for the categorization of clinical *A. baumannii* isolates, showing a concordance of 98.2% with BMD, due to the fact that two very major errors were found (1.7%). Therefore, we suggest that E-test results should be confirmed by a dilution method, especially when colistin use is required for the treatment of serious systemic infections caused by *A. baumannii* and when the MIC is determined to be 1 to 2 µg/ml, a range where we have found the two very major categorical discrepancies.

Garnacho-Montero et al. (9) demonstrated that intravenous colistin was as effective as imipenem in an open evaluation of the treatment of 21 episodes of ventilator-associated pneumonia caused by multidrug-resistant *A. baumannii*. Jiménez-Mejías et al. (11) reported successful treatment of a case of multidrug-resistant *A. baumannii* meningitis by using intravenous colistimethate. Colistimethate was the only antibiotic to which the *A. baumannii* strain was susceptible (MIC, 0.3 µg/ml). These two studies show the clinical correlation of the two susceptibility tests in the detection of colistin resistance in *A. baumannii* clinical isolates, because all the strains isolated in both studies are included in our report.

In summary, the results of the present study suggest that the E-test could be a reliable and suitable alternative to the reference method for the detection of colistin resistance in *A. baumannii* clinical isolates by clinical laboratories. However, the poor agreement found for the susceptible strains for which MICs were lower deserves additional consideration in relation

to the pharmacokinetics and pharmacodynamics of colistin in the treatment of severe infections.

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REFERENCES

1. Appleman, M. D., H. Belzberg, D. M. Citron, P. N. Heseltine, A. E. Yellin, J. Murray, and T. V. Berne. 2000. In vitro activities of nontraditional antimicrobials against multidrug-resistant *Acinetobacter baumannii* strains isolated in an intensive care unit outbreak. *Antimicrob. Agents Chemother.* **44**:1035–1040.
2. Arroyo, L., D. Martín-Lozano, A. García-Curiel, M. Pachón-Ibáñez, A. Llanos, M. Ruiz, J. Aznar, and J. Pachón. 2003. Correlation between broth microdilution and E-test methods in the MIC determination of colistin in clinical *Acinetobacter baumannii* strains, abstr. P762, p. 168. Program Abstr. 13th Eur. Congr. Clin. Microbiol. Infect. Dis. European Society of Clinical Microbiology and Infectious Diseases, Basel, Switzerland.
3. Bergogne-Berezin, E., and K. J. Towner. 1996. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clin. Microbiol. Rev.* **9**:148–165.
4. Catchpole, C. R., J. M. Andrews, N. Brenwald, and R. Wise. 1997. A reassessment of the in vitro activity of colistin sulphomethate. *J. Antimicrob. Chemother.* **39**:255–260.
5. Evans, M. E., D. J. Feola, and R. P. Rapp. 1999. Polymyxin B sulfate and colistin: old antibiotics for emerging multidrug-resistant gram-negative bacteria. *Ann. Pharmacother.* **33**:960–967.
6. Fernandez-Viladrich, P., X. Corbella, L. Corral, F. Tubau, and A. Mateu. 1999. Successful treatment of ventriculitis due to carbapenem-resistant *Acinetobacter baumannii* with intraventricular colistin sulfomethate sodium. *Clin. Infect. Dis.* **28**:916–917.
7. Fluit, A. C., M. E. Jones, F. J. Schmitz, J. Acar, R. Gupta, and J. Verhoef. 2000. Antimicrobial susceptibility and frequency of occurrence of clinical blood isolates in Europe from the SENTRY antimicrobial surveillance program, 1997 and 1998. *Clin. Infect. Dis.* **30**:454–460.
8. Gales, A. C., A. O. Reis, and R. N. Jones. 2001. Contemporary assessment of antimicrobial susceptibility testing methods for polymyxin B and colistin: review of available interpretative criteria and quality control guidelines. *J. Clin. Microbiol.* **39**:183–190.
9. Garnacho-Montero, J., C. Ortiz-Leyba, F. J. Jiménez-Jiménez, A. E. Barrero-Almodovar, J. L. García-Garmendia, M. Bernabeu-Wittel, S. L. Gallego-Lara, and J. Madrazo-Osuna. 2003. Treatment of multidrug-resistant *Acinetobacter baumannii* ventilator-associated pneumonia (VAP) with intravenous colistin: a comparison with imipenem-susceptible VAP. *Clin. Infect. Dis.* **36**:1111–1118.
10. Go, E. S., C. Urban, J. Burns, B. Kreiswirth, W. Eisner, N. Mariano, K. Mosinka-Snipas, and J. J. Rahal. 1994. Clinical and molecular epidemiology of *Acinetobacter* infections sensitive only to polymyxin B and sulbactam. *Lancet* **344**:1329–1332.
11. Jiménez-Mejías, M. E., C. Pichardo-Guerrero, F. J. Marquez-Rivas, D. Martín-Lozano, T. Prados, and J. Pachón. 2002. Cerebrospinal fluid penetration and pharmacokinetic/pharmacodynamic parameters of intravenously administered colistin in a case of multidrug-resistant *Acinetobacter baumannii* meningitis. *Eur. J. Clin. Microbiol. Infect. Dis.* **21**:212–214.
12. Levin, A. S., A. A. Barone, J. Penco, M. V. Santos, I. S. Marinho, E. A. Arruda, E. I. Manrique, and S. F. Costa. 1999. Intravenous colistin as therapy for nosocomial infections caused by multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. *Clin. Infect. Dis.* **28**:1008–1011.
13. Manikal, V. M., D. Landman, G. Saurina, E. Oydna, H. Lal, and J. Quale. 2000. Endemic carbapenem-resistant *Acinetobacter* species in Brooklyn, New York: citywide prevalence, interinstitutional spread, and relation to antibiotics usage. *Clin. Infect. Dis.* **31**:101–106.
14. National Committee for Clinical Laboratory Standards. 2003. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th ed. Approved standard M7–A6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
15. Reis, A. O., D. A. Luz, M. C. Tognim, H. S. Sader, and A. C. Gales. 2003. Polymyxin-resistant *Acinetobacter* spp. isolates: what is next? *Emerg. Infect. Dis.* **9**:1025–1027.
16. Urban, C., N. Mariano, J. J. Rahal, E. Tay, C. Ponio, T. T. Koprivnjak, and Jerrold Weiss. 2001. Polymyxin B-resistant *Acinetobacter baumannii* clinical isolate susceptible to recombinant BP121 and cecropin P1. *Antimicrob. Agents Chemother.* **45**:994–995.
17. Vaneechoutte, M., L. Dijkshoorn, I. Tjernberg, A. Elaichouni, P. de Vos, G. Claeys, and G. Verschraegen. 1995. Identification of *Acinetobacter* genomic species by amplified ribosomal DNA restriction analysis. *J. Clin. Microbiol.* **33**:11–15.
18. Vassen, W., P. Desmery, S. Ilutovich, and A. Di Martino. 2000. Intrathecal use of colistin. *J. Clin. Microbiol.* **38**:3523.